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14. ABSTRACT

Many Gulf War (GW) veterans experience chronic symptoms of Gulf War Illness (GWI), Although GWI remains a disorder primarily diagnosed by self-report of symptoms, our research has shown important preliminary evidence for brain imaging biomarkers correlated with these symptoms. This study builds on our key Boston Gulf War Illness Consortium (GWIC) multi-modal brain imaging findings in GWI cases that 1) white matter (WM) microstructural alterations on diffusion tensor imaging (DTI) are present and related to behavioral outcomes, 2) functional connectivity alterations are present on fMRI and pCASL, and 3) Brain volumes are reduced in gray matter (GM) and WM pathways. The study objectives are to confirm, validate and further define WM microstructural integrity decrements in multiple imaging modalities (DKI and HARDI). This proposal aims to assess the overlap of WM decrements with cerebral blood alterations (pCASL) in GWI cases vs controls. We hypothesize that GWIC study will be confirmed and perhaps worsened on longitudinal analyses in GWI cases. Lastly, data reduction techniques will be employed to predict GWI case status, identify clinically relevant subgroups and changes in illness presentation over time by utilizing machine learning analytics of DKI, diffusion MRI, pCASL and brain volumetric analyses in this proposed longitudinal dataset.

15. SUBJECT TERMS
Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, pesticides, DFP, sarin

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Table of Contents

Page

1. INTRODUCTION:

The study objectives are to confirm, validate and further define white matter microstructural integrity decrements in multiple neuroimaging modalities (DKI and HARDI). This proposal also aims to assess the overlap of white matter decrements with cerebral blood flow alterations (pCASL) in Gulf War Illness cases vs controls. We hypothesize that GWIC findings of lower gray matter and white matter volumes and microstructural integrity will be confirmed and perhaps worsened on longitudinal analyses in Gulf War illness cases. Lastly, data reduction techniques will be employed to predict Gulf War illness case status, identify clinically-relevant subgroups and changes in illness presentation over time by utilizing machine learning analytics of DKI, diffusion MRI, pCASL and brain volumetric analyses in this proposed longitudinal dataset.

2. KEYWORDS:

Gulf War Illness, GW veterans, neuroimaging, biomarkers, white matter, cerebral blood flow

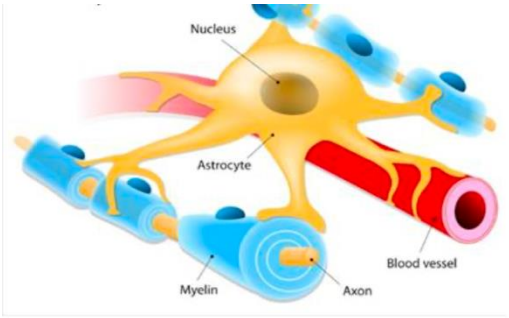
3. OVERALL PROJECT SUMMARY:

INTRODUCTION

Statement of the Problem. Veterans of the 1991 Gulf War (GW) continue to experience chronic symptoms, including fatigue, memory and concentration problems, muscle and joint pain and headaches known as GW Illness (GWI). Brain white matter (WM) alterations have now been shown to be present in veterans with GWI in several different studies suggesting a pathobiological link to the disorder.[1,2,3,4,19]. Myelinated brain WM is highly susceptible to lipophilic neurotoxicants because it is made up of nearly 80% lipids. WM changes have been correlated with neurotoxicant exposures during the war in GW veterans including the acetylcholinergic inhibiting (AChEi) nerve agent sarin and similarly acting pesticides[1,6,2,3,4]. Progress in understanding how AChEi's affect the CNS has made tremendous strides due to work from the Boston GWI consortium and other GWI investigators. What is now known, is that acute GW-relevant AChEi organophosphate (OP) exposures not only affect AChE but also cause neuroinflammation, oxidative stress, axonal transport deficits and directly affect myelin structure as well as oligendrocyte proliferation[7,8,9,10,11]. However, ***the mechanisms for chronic WM alterations in veterans with GWI and whether it is static or progressively getting worse remains unknown***. Parsing out this question is crucial because WM damage can cause changes to local gray matter (GM) structures and brain vasculature and vice- versa. It is unknown if these WM changes are progressive over time and getting worse as veterans age and are less able to compensate for them or if GWI has become a progressive neurodegenerative disorder outside of normal aging parameters. Answering these questions with longitudinal brain imaging and behavioral correlation analyses is crucial to determining appropriate treatment strategies based on the true course of the disorder.

Background. Using existing and newly collected longitudinal data from our large multi-site consortia studies, the proposed project will build on our current findings of central nervous system (CNS) WM structural alterations in GWI, characterize perfusion abnormalities in GWI, and test an empirically developed algorithm for identifying GWI case status and clinically-relevant subgroups of the disorder over time. The CNS is made up of neurons and glia. The three types of glia include oligodendroctyes, astrocytes and microglia. Oligodendrocytes, glial cells that make myelin sheaths, are considered the WM of the brain. Efficient transfer of information between brain regions only occurs with intact WM functioning. WM allows for fast transmission of information across neuronal synapses and altered WM pathways can result in delayed information processing speed and efficiency. WM is highly susceptible to both neurotoxicant exposures and to microvascular changes[12]. WM damage with microvascular

components can result in ischemic stroke and/or dementia[12]. Cognitive decrements have been shown to correlate with WM alterations on brain imaging in the Boston Gulf War Illness Consortium (GWIC)[13]. Microglia and astrocytes are considered the innate immune sentinels of the CNS[5]. Oligodendrocytes have several kinds of neurotransmitter receptors, including those for acetylcholine and glutamate[5]. GWIC related studies now show that GW-relevant organophosphates (sarin, pesticides) affect myelination by impairing neurotransmitter function in oligodendrocytes. Glial cells greatly outnumber neurons in the CNS and astrocytes are the most common form of glia. Astrocytes not only support neurons but they also completely encapsulate the neuronal synapse directly interacting with neurons and altering neuronal communications because of the direct contact with the synapse through astrocytes releasing neurotransmitters that also affect neurons. Astrocytes also protect neurons from foreign invaders by processing toxins, cellular debris and excitatory neurotransmitters through phagocytic processes. In addition, ***astrocytes directly interact with blood vessels causing them to expand or narrow and affecting the flow of oxygen and nutrients***. ***Astrocytes are also intimately involved in blood brain barrier (BBB) function.*** Astrocytes and microglia are activated by the excitatory neurotransmitter glutamate and release proinflammatory cytokines and chemokines which can cause neuroinflammation. We have also reported blood markers of CNS proteins, including myelin basic protein (MBP), neurofilament triplet protein (NFP), tau, microtubule associate protein (MAP-2), glial fibrillary acidic protein (GFAP) and calcium-calmodulin Kinase II (CAMKII), are increased in veterans with GWI[14]. This change in CNS proteins indicate damage to the neuronal cytoskeleton, axons and glial alterations including astroctyes[15,16,17]. Detecting these CNS protein autoantibodies in the blood where they are not usually found, suggests a leaky blood brain barrier (BBB) was present (at least previously) releasing these proteins into the periphery and causing the autoantibodies to be formed. Collectively, ***these findings suggest that microvascular changes may also be added to the list of AChEi effects***. It has specifically been found in our GWIC studies that higher blood levels of these CNS proteins as well as glutamate excitatory neurotransmitter and circulating phosphate levels correlate with lower brain WM volumes and impaired microstructural integrity on diffusion tensor imaging (DTI) in veterans with GWI (see preliminary results). Thus promising leads for objective biomarkers of GWI are becoming evident. However, concerns regarding altered BBB permeability and declining cerebrovascular health as well as worsening WM damage as GW veterans' age is mounting instigating this proposal for longitudinal assessment of WM in ailing GW veterans.



Specifically, clearance of myelin debris from damaged WM in the CNS can take years to complete due to Wallerian degeneration or die-back mechanisms that begin at the site of injury, followed by degeneration and phagocytosis of axons and myelin further from the initial site of injury. Myelin clearance in the CNS can be very slow. Microglia are involved in Wallerian degeneration and once activated, can act as phagocytes to eliminate myelin debris similar to macrophages in the peripheral nervous system. This can cause signaling and activation of nearby microglia, astrocytes and neurons. Additional microglial and astrocyte activation (priming) and subsequent neuroinflammatory responses due to myelin or neuronal breakdown products in the extracellular spaces are considered danger signals to the microglia and can result in chronic glial activation loops. For example, studies in rats have shown that myelin sheaths remained in the CNS *for almost 2 years* after axonal degeneration. This could correspond to *decades in humans*. Microglial priming and chronic glial activation loops tend to occur more commonly in advanced aging and in neurodegenerative states suggesting this possibility in veterans with GWI.

Neuroinflammation and oxidative stress often occur in tandem in OP exposures and can exacerbate CNS damage[11]. Oxidative stress related reactive oxygen species (ROS) can cause ischemic vascular changes to the CNS[18] including damage to lipids and cell membranes. ROS have been shown to alter vascular

functions including BBB permeability and to cause ischemic lesions[18]

Although the finding that cerebral WM injury has been reported multiple times in GWI research, until now there has not been mechanistic hypotheses for these findings[1,2,19,20]. It is possible that the findings of increased CNS autoantibodies in peripheral blood of ill GW veterans is suggestive of a leaky blood brain barrier (BBB) resulting in the release of these proteins in to the periphery and reducing cerebrovascular health. However, there have been very few studies assessing cerebrovascular health, including the hemodynamic response of blood flow and BBB integrity in veterans with GWI. One recent study conducted by Falvo et al. found significantly impaired dynamic cerebral autoregulation from sitting to standing in veterans with GWI relative to those without, comparable to the level of impairment observed in patients with carotid artery stenosis and ischemic stroke[21]. Similarly, the Rotterdam Aging Study reported that ever global WM hyperintensity (WMH) increment was associated with a higher incremental risk for stroke and mortality[30]. In the animal literature it has been shown that it is possible to perform MRI measures of BBB permeability in ischemic stroke using diffusion-weighted arterial spin labeling in rats[22]. Correspondingly, absolute perfusion quantification allows for recognition of global hypo- or hyper-perfusion states and also permits comparison between multiple measurements in longitudinal studies[23]. Combining structural, microstructural and functional neuroimaging holds promise for answering these questions regarding neural networks in GWI. DTI can identify and characterize the WM tracts that provide the neuroanatomic connectivity between GM structures identified by functional MRI (fMRI).

Altered cerebral blood flow (CBF), glutamate and calcium signaling have also been associated with GW-relevant organophosphate (OP) exposures[24,26,26]. Reported of OP exposed flight crew members where WM microstructural integrity impairments were reported in corpus callosum pathways and CBF was reportedly altered in the prefrontal cortex and the precuneus on arterial spin labeling (ASL) imaging provide important clues for GWI pathology because these results are remarkably similar to GWIC brain imaging results to date[27]. One study to date in GW- relevant sarin exposed rats has shown reduced heart rate variability suggesting neurotoxicant induced altered cerebrovascular functioning[28]. In addition, circulating phosphate levels have recently been associated with WM hyperintensities (WMH) and microstructural integrity alterations associated with small vessel cerebrovascular disease[29]. In fact, recent reports suggest that ***microstructural WM changes are detected before noticeable small- vessel cerebrovascular disease thus providing the potential to detect these early changes perhaps in time to intervene*** and treat them in veterans at-risk for cerebral small vessel disease[30]. High phosphate has also been shown to induce apoptosis in endothelial cell lines and the effect was enhanced when calcium was added[7]. The process of phosphate-induced apoptosis was further characterized by increased oxidative stress, as detected by increased ROS generation and disruption of the mitochondrial membrane potential[7]. These findings showed that high phosphate levels can cause endothelial cell apoptosis, a process that impairs endothelial integrity which could be important to both BBB function and to potential vascular disease[7].

WM and CBF alterations often co-occur. Higher axial diffusivity measures have been reported in some WM major fiber pathways in the brains of GW veterans exposed to neurotoxic agent[27,15]. However, some studies have shown higher CBF in some regions with WMH while others have shown lower CBF perhaps indicating compensatory mechanisms in some areas for other less functional areas. Increased phosphate levels have also been linked with vascular calcification and endothelial dysfunction as well as cerebral small vessel disease[29]. Cerebral small vessel disease affects all major blood vessels including arterioles, capillaries and venules which can lead to changes in brain WM integrity and structural mophometry[29.] Small vessel disease in WM microstructural DTI studies often shows as a pattern of decreased fractional anisotropy (FA) values and increased mean diffusivity (MD) values[30]. It is important to note that altered WM microstructural integrity can progress to the more serious white matter hyperintensities (WMH) over time and when this occurs, it suggests far greater risk for significant cerebrovascsular sequelae including

stroke, dementia and mortality risk. Because DI is more sensitive to subtle WM microstructural injury than WMH, it provides an opportunity to assess change over time in veterans with GWI perhaps before more clinically significant changes have occured[30,39].

This study is designed as a call-back study to the BBRAIN repository of study participants who are also GWIC participants in the Boston and Houston sites. These veterans will come back for their Time 2 brain scans as part of this study. This will enable us to compare longitudinal WM, WMH and CBF over time in these veterans over a 3-4 year time period. These results will be compared with blood markers and cognitive outcomes from the BBRAIN repository collected in close time proximity to the Time 2 brain scans. Brain imaging data from this study will be included in the BBRAIN brain imaging repository as a unique longitudinal imaging resource for GWI researchers.

Objectives: Study objectives are to confirm, validate and further define WM microstructural integrity decrements in multiple imaging modalities (DKI and HARDI). The proposal also aims to assess the overlap of WM decrements with GM CBF alterations (pCASL) in GWI cases vs controls. We hypothesize that GWIC findings of lower GM and WM volumes and microstructural integrity will be confirmed and perhaps worsened on longitudinal analyses in GWI cases. Lastly, data reduction techniques will be employed to predict and validate GWI case status, identify clinically-relevant subgroups and changes in illness presentation over time by utilizing machine learning analytics of DKI, diffusion MRI, pCASL and volumetric analyses in this longitudinal dataset.

Research Strategy:

Utilizing resources from the Gulf War Illness Consortium and BBRAIN Repository: The team comprising the Boston Biorepository, Recruitment and Innovative Network (BBRAIN) for GWI has expertise and experience in initiating and successfully implementing a GWI consortium (GWIC), a precursor to the BBRAIN biorepository. The Boston GWIC (GW120037) is an international 9-site collaboration which has created a multi-site electronic data capture platform for blood, saliva, cognitive, brain imaging and health symptom data for 300 study participants (200 GWI cases, 100 controls). The GWIC has undertaken a coordinated series of clinical (3 sites) and preclinical studies to comprehensively understand the pathobiology of GWI. This includes clinical studies that have brain imaging scans (Time 1), neuropsychological testing data, and immune and genetic measures. Parallel preclinical studies have evaluated persistent effects of GW neurotoxicants in vitro and in rodent models of GWI showing effects on both axonal function and oligodendrocyte function and neuroinflammation with GW relevant OP exposures[5,10,15]. The GWIC is led by Dr. Kimberly Sullivan at Boston University Medical Campus (BUMC) with additional clinical site directors and investigators at Nova Southeastern University (Drs. Klimas, Abreu) and Baylor College of Medicine (Drs. Steele, Little). In the GWIC studies, cases and controls are determined by Kansas GWI criteria but CDC GWI criteria is also collected for comparison purposes[36,37]. The neuroimaging and machine learning studies are conducted by BU investigators (Drs. Killiany, Koo, Palumbo).

Utilizing and Contributing to the BBRAIN Multi-center Neuroimaging Data Informatics Repository – BBRAIN also includes a multi-center imaging data repository that is being combined and applied for reconstruction as an informatics repository by Dr. Koo at BUMC. Dr. Koo will apply the most advanced quantitative brain mapping strategies to T1/T2 structural MRI, diffusion MRI, resting-state functional MRI and pulsed arterial spin labeling (pCASL) to the current study. Here, brain mapping strategies include cortical thickness/shape analysis, hippocampal subfield analysis, cortical micro-structure analysis, white matter major fiber extraction, structural and functional network mapping and tracer mapping. Based on these indices, we will build longitudinal machine- learning and deep learning based multi-stage data classification system to provide underlying neurological information on GWI to the Boston BBRAIN

participants. Since many of the collected neuroimaging data have paired information on non-imaging bio-measurements such as blood cytokines and cognitive tests, the classification system will also contain information on canonical relationship between imaging and non-imaging measures including phosphate, glutamate and cytokine markers. This longitudinal data informatics repository will be distinct from the BBRAIN data repository and will be shared with other BBRAIN and GWI investigators.

Study Participants. *Participant Demographics* – Former GWIC study participants will be recruited for the BBRAIN study and for this study as a call-back to BBRAIN. GWIC study participants have an average age of 52 years, 15 years of education, 75% Caucasian and include 15% women. Demographics are not significantly different between cases and controls. Nearly 200 GWIC participants have been recruited to date and 500 participants will be recruited for BBRAIN. Brain imaging was done in the Boston and Houston sites for the GWIC and 100 participants will be recruited for this main study in Boston and 5 participants will be recruited for the perfusion validation study at the Houston site.

Definition of GWI – GWI case status is defined by the Kansas GWI case defintion[36]. The definition requires GWI cases to endorse multiple or moderate-to-severe chronic symptoms in at least three of six statistically- defined symptom domains: fatigue/sleep problems, somatic pain, neurological cognitive, mood symptoms, gastrointestinal symptoms, respiratory symptoms and skin abnormalities. Veterans are excluded from being considered GWI cases, for purposes of the research study, if they report being diagnosed by a physician with medical or psychiatric conditions that would account for their symptoms or interfere with their ability to report their symptoms. Potential participants will be screened by telephone to determine whether they still meet inclusion or exclusionary criteria for study participation. For comparison purposes, veterans will also be categorized for Fukuda et al. CDC criteria[37]. Specific exclusion criteria include such medical conditions as diabetes, heart disease other than hypertension, stroke, lupus, multiple sclerosis, cancer, liver disease, chronic infection, or serious brain injury. Veterans are also excluded if they report being diagnosed with schizophrenia or bipolar disorder or if they have been hospitalized in the past 5 years for alcohol/drug dependence, depression, or post-traumatic stress disorder (PTSD). Veterans with current or past history of PTSD or depression in the past 5 years will not be excluded if they were not hospitalized for these conditions in the 5 years before the study.

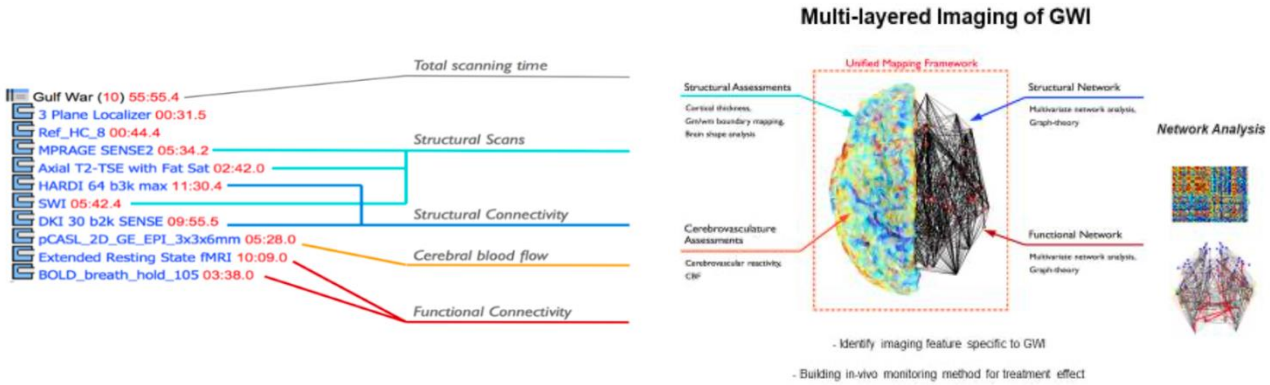
Recruitment Process and Informed Consent – Eligible GW veteran cases and controls will be recruited at BUMC by mail and telephone contacts with veterans who have previously participated in the GWIC study. Eligible GW veteran cases and controls will be recruited at the Boston site by mail and telephone contacts with veterans who have previously participated in the GWIC study by re-contacting this 175 member GW veteran cohort through re- contacting participants who have agreed to be contacted for future studies. The initial contact will be performed by the PI or a PI designated staff member. Eligible participants will be given a description of the study protocol including brain imaging and basic clinical examination (blood pressure etc.) survey, time required, and reimbursement for their time and effort. Subjects will be given a local telephone number and will have an opportunity to ask questions about the procedures. They will be informed that their decision to or not to participate will have no bearing on their medical care and that, if they choose to participate, they may withdraw at any time without loss of any medical or University benefits to which they are entitled. Informed consent will be obtained by the study investigator or by an IRB-approved delegate after a screening interview, but prior to the initiation of any study procedures. As the initial step in the consent process, the study will be further explained to the veteran in detail and the consent form will be reviewed with the veteran in its entirety by the PI or an IRB- approved delegate. The participant will be fully informed about all aspects of the study and will be given ample time for clarification and to have his or her questions answered. The GW veteran will be able to bring the consent form home and discuss the study with their family members, or others prior to making a decision.

Sharing Study Results - The brain imaging tests that are completed as part of this research project may uncover medical problems that the research participant was not previously aware of. As such, if a clinically significant medical abnormality is detected, the results of this study will be shared with the human subjects and, with their consent, their primary care provider. If an urgent medical situation is detected, the participant will be escorted to the emergency room for emergency care.

Study Methods: Our GWIC brain imaging protocol is listed below and was designed to assess all major aspects of brain health suspected to be associated with GWI and neurotoxicant exposures. These include structural imaging, microstructural integrity measures, brain flow and hemodynamics and functional integrity. The proposed study will provide a longitudinal analysis using the same imaging protocol and the same GWIC participants to further validate and quantify the prior findings and to compare any further loss of volume/microstructural integrity with quantified hemodynamic response integrity. We propose the same protocol with multimodal magnetic resonance imaging (MRI) listed below in 100 GW veterans (50 GWI cases, 50 controls). All participants will be recruited for the newly funded Boston Biorepository and Integrated Network (BBRAIN) for GWI where they will also undergo cognitive assessments, provide blood, saliva, urine and other relevant biomarkers to share for GWI studies. This proposed study will be a BBRAIN call-back study and the MRI data will be included in the BBRAIN repository. Although our plan is to recruit the first participants from GWIC as the first BBRAIN participants with a four-year time frame, it is unlikely that we will be able to match the exact 4 year time interval between the MRI scan previously acquired in the original GWIC study and the one acquired longitudinally here between the participants. To control for this potential time difference issue and to further quantify change over time, the longitudinal data will be expressed as *an annualized change* (time 1 – time 2/days between scans/365)[39].

MRI Imaging: Each of the former GWIC participants from the Boston site will undergo MRI scanning on a 3 Tesla Philips scanner at the Boston University Center for Biomedical Imaging (CBI) where Dr. Killiany is Imaging Director for this follow-up longitudinal study. The scanning session will include: 1) Three plane TFSE scout scan (*TR=11; TE=2.4; TFE Shots=2; Matrix=256x128; Voxel=0.98x1.95x10mm^3*), 2) a Sense reference Scan (*TR=4.0; TE=0.75; TFE Shots=2; Matrix=96x75; Voxel=5.52x7.07x6.0mm^3*), 3) an accelerated high resolution MPRAGE scan acquired in the sagittal plane (*TR=6.8; TE=3.1; TFE=233; T1 Delay=823; B1 rms= 0.75uT; Matrix=244x227; Slices=170; Orientation= Sagittal; Voxel=1.11x1.11x1.2mm^3*), 4) a T2-weighted scan acquired in the axial plane (*TR=3000; TE=80; TSE=10.0/150; Matrix=256x255; Slices=45; Orientation= Axial; TSE Factor=15; Fat Suppression=SPIR; Voxel=.94x.94x4mm^3*, 5) a Diffusion Scan with a B0 and 2 b-values (1000 & 2000) and 30 encoding direction per each b value *(TR=9834; TE=74; Matrix:96x96; Voxel=2.5x2.5x2.5mm^3; DELTA: 36.6/15.2; EPI Factor: 51; Directions: 30*, 6) a resting state functional magnetic resonance imaging scan *(TR=3000; TE=30; Matrix: 64x64; Voxel: 3.3x3.3x3.3; Slides=48 (no gap),* and 7) a pCASL sequence obtained while the participant is at rest (*TR=4000; TE=11; Matrix: 64x64; Voxel: 2.75/2.75/5; Slices=20; FOV=220x220x119; Label Type: Parallel Slab; Gap: 30.5; Label Duration: 1500; Label Delay: 1500* 8) a High Angular Resolution Diffusion Imaging (DTI) scan with a B0, B3000 and 64 encoding directions *(TR=8118; TE=100; Matrix:112x112; Voxel=2x2x2mm^3; DELTA: 49.8/33.9; Directions: 64).*

Brain Imaging Post-processing: The MRI scans will be transferred electronically in DICOM format to our research PACS system located at the Center for Biomedical Imaging at Boston University School of Medicine. At the time of acquisition, each scan will undergo quality checking that consists of a visual inspection for the presence of noise or artifact as well as a review of scan parameters to ensure that the appropriate ones were used in the acquisition. Scans that pass the quality check will be entered the post-processing pipeline.



MPRAGE: Post-process of the MPRAGE scans will be conducted using the software "Freesurfer" that has been developed for free distribution by Drs. Dale and Fischl of the Martinos Center at Massachusetts General Hospital. We have worked closely with Drs. Dale and Fischl to develop the atlas that is used to subdivide the brain into regions of anatomical interest. Each scan is first corrected for motion, averaged, normalized for intensity before it is r-sampled to isotropic dimensions of 1*1*1 mm using previously published algorithms that are distributed in the FreeSurfer software package. Next, the skull is removed from the images using a skull-stripping algorithm and the images are segmented to identify the dorsal, ventral and lateral extent of the gray/white matter boundary. This provides a surface representation of the cortical WM and is a more accurate task for automated programs than attempting to find the gray matter boundary on the surface of the brain. The quality of the skull stripping and accuracy of the gray/WM boundary for each subject is reviewed by anatomically skilled operators. The cortical WM surfaces generated by the steps above are automatically corrected for topological defects and thereafter utilized in a deformation procedure that locates the pial (GM) surface of the brain. The output of the Freesurfer program contains measure of volume, cortical thickness and surface area for a number of anatomical structures of interest that will serve as regions for the other forms of image processing. Importantly for this grant, *the Freesurfer processing pipeline has a specific mechanism for managing longitudinal analyses*. Each T1 weighted MRI scan will be processed independently. Once all steps are completed, then the scans from the two time points are run through the final longitudinal Freesurfer processing pipeline to ensure the date is treated appropriately. ***Multi B sphell diffusion scan***: We will be obtaining a multi B shell diffusion scan (HARDI and DKI) on all participants. The information from this scan will be used to assess the neurite orientation and dispersion properties of the gray and white matter of the brain using the NODDI technique[38]. measures of orientation dispersion (OD) throughout the brain. OD maps will be co-registered with the Freesurfer generated maps of regions of interest in the gray matter and the various WM pathways that were generated from the Freesurfer tracula pipeline. Average and standard deviation OD measures from each of these brain regions can then be used to assess for changes over time.
Multi-spectral image based WM hyperintensity segmentation: We will combine T1, T2-weighted scan and multiparametric diffusion map to segment WM hyperintensity components using in-house processing scripts. T2 weighted scans have better sensitivity to detect hyperintensities than T1 scans. Also, diffusion imaging (MD, free- water diffusivity or hindered diffusion components) could have additional sensitivity to detect perivascular leakage in the brain[39]. The script combines multispectral MRI scans and runs k-nearest neighbor classifications. Reconstructed hyperintensity maps will be combined to the WM major tracts to generate regional WM hyperintensity quantifications.
HARDI: HARDI scan analysis will be conducted in two ways. First we will use a structural network analysis scheme based on graph theory that allows for assessment of direct and indirect connections providing a reliable way to quantify brain networks with a small number of neurobiologically meaningful and easily computable measures.
Structural network analysis: Seventy-eight transformed GM regions of interest from the Freesurfer processed MPRAGE scans (Desikan-Killiany atlas) will be used for seed and target regions for WM

fiber-tracking. We will use a probabilistic fiber tracking method with structure masks that allow for *proper estimation of complicated fiber crossings*. Longitudinal between group comparisons will be performed on quantitative network measures: 1) whole brain connectional efficiency, 2) hemispheric connectional efficiency, 3) nodal efficiency and degree on each node (ROIs). The number of neighbors of the node is defined as the degree (strength) and reflects importance of nodes in the network. Sequences of distinct nodes and links in anatomical networks can be defined as the path and represent potential routes of information flow between pairs of brain regions. Lengths of paths consequently estimate the potential for functional integration between brain regions, with shorter paths implying stronger potential for integration. 'Efficiency' is defined as inverse of Lengths of paths. In-House made Matlab (Mathworks) scripts based on Brain connectivity toolbox will be applied for the computations on these indices.

Freesurfer tracula analysis: One of the shortcomings of the structural network analysis described above is that it does not necessarily give you a good measure of the same "established" pathway in each subject. In order address this concern, we will also be processing the HARDI data (in combination with the MPRAGE data) through the Freesurfer tracula pipeline. This technique using a probabilistic mapping method to identify a set number of known pathways in the brain. Individual subject special maps in individual subject space are generated. From these maps we can assess measures such as average and standard deviations of known diffusion measures such as fractional anisotropy (FA) and radial diffusivity (RD). Further, these maps can be co-registered with other imaging maps. In this study, we will use these maps to ensure that we have diffusion information and neurite density measures from the exact same regions in each subject.

Resting State fMRI: Fluctuations in the blood oxygenation level-dependent (BOLD) signal during rest has been postulated to provide information about the functional interactions between different brain regions. In this project, we will use functional network analyses method using in-house developed processing pipeline. Processing steps include non-brain voxel extraction, geometric distortion correction, intra-volume realign, inter-volume realign and nonlinear noise reduction, motion correction, spatial smoothing. After preprocessing, the T1 structural scans of each subject will be co-registered to average rsfMRI volume. The same co-registration parameters will be then applied to transform the 78 gray matter defined regions of interest from FreeSurfer. Functional connectivity is defined as the temporal correlations between the various gray regions of interest. The correlation between a given pair of regions is typically used to index functional connectivity and threshold is applied to the resultant pairwise correlation matrices to generate a statistically significant functional connectivity network. Permutation based random effect corrections will be used for multiple comparison corrections. The number of permutations will be 10,000 for correcting possible random effects on each correlation case. After calculating the correlation between each pair of brain regions functional brain complex network systems will be represented in a two dimensional connectivity matrix forms. Brain connectivity toolbox will be used for computations on brain network measures.

pCASL: Assessment of Arterial Blood flow: We will also be measuring resting state blood flow using a pseudo- Continuous Arterial Spin Labeling (p-CASL) sequence. This sequence combines the advantages of a Continuous- Arterial Spin Labeling sequence with a Pulsed-Arterial Spin Labeling sequence. In particular the pCASL sequence was developed to provide the sensitivity of a continuous arterial spin labeling procedure while overcoming many of the shortcomings of a continuous acquisition. This sequence allows one to detect differences in CBF using non- invasive procedures. The scan will be acquired with the subject at rest in the scanner. As with the resting state fMRI scan, FMRIB's software toolkit will be used for preprocessing of the p-CASL scans including non-brain voxel extraction and geometric distortion correction. After preprocessing, the MPRAGE scans of each subject will be co-registered to p-CASL volume. The same co-registration parameters will be then applied to transform the 78 ROIs defined from FreeSurfer analysis of the MPRAGE scan in order to define the same regions in the p-CASL space so that measures of arterial blood flow from the various gray matter and white matter ROI

can be assessed separately.

Machine Learning Classifier: The above independent measures and their annualized change values will produce different types of feature pools, which form a basis information on building the machine learning classifiers. To build a computer-aided classifier of GWI, we will employ random decision forest classification (RDF). RDF performs iterative partitioning of the multivariate feature space to identify decision boundaries that highlight differences between groups. RDF also provides feature importance weights to each of the features selected for the classification and allow us to identify which combinations provide the best sensitivity for the group comparisons.

Perfusion Validation Sub-study. Dr. Little will collaborate with Drs. Sullivan and Killiany on design and imaging analysis and will have primary responsibility for the perfusion validation aims including data collection, analysis, integration with ASL and evaluation of cortical volumes and thickness. While pCASL is a state of the art measure it is an indirect method to quantify perfusion abnormalities. Because the nature of the perfusion abnormality is unknown in GWI a validation is absolutely critical. To do so, we propose to collect a small amount of MRI perfusions data on GW veterans who have previously consented to being re-contacted for future studies. Dynamic susceptibility contrast-enhanced MR perfusion is a high temporal resolution technique during which the bolus of gadolinium-based contrast agent is injected into an IV with the participant on the MRI table and in the MRI. A T1 weighted MPRAGE sequence will be collected first. The gadolinium will then be injected while T2* weighted imaging (the same idea behind the resting state functional MRI already described) is collected. The gadolinium causes a susceptibility effect because the contrast agent is paramagnetic which causes the MRI signal to drop. This reduction in signal will then be converted to a signal intensity time curve on a voxel-by-voxel basis. This can then be converted to parametric maps of CBF which can be analyzed using the same ROI methods described above. Direct comparisons between pCASL and perfusion MRI (pMRI) will allow an assessment of validation of blood flow. This data will be collected in Houston at the Center for Advanced Imaging at the University of Texas Houston Science Center located in the Texas Medical Center of which both UT and BCM are members allowing use of systems across institutions. The UTHSC system is focused on perfusion imaging with all necessary hardware and software for analysis. Data collection will be carried out on the 3 Tesla Siemens Prisma MRI at UTHSC. This magnet and imaging center have a specific focus on MRI-based perfusion studies and are fully equipped to integrate millisecond accuracy of the bolus of gadolinium allowing integration of physiology on the same scale as pCASL. This magnet has similar gradient characteristics as the Philips 3T MRI scanner that we are using in Boston for the remainder of the longitudinal studies. As such, the Houston scanner is able to run an equivalent pCASL sequence as the one used at the Boston site. This will allow us to examine perfusion abnormalities and interpret the perfusion data accurately in this population. In this validation sub-study, 5 GW veterans in Houston will undergo perfusion studies twice over two years for a longitudinal measure of perfusion outcomes.

Neuropsychological Measures- Measures from a previously validated assessment of cognitive function in GW veterans will be included to assess for longitudinal changes in cognitive outcomes[31,32,33]. The neuropsychological test battery from GWIC and BBRAIN assesses the functional domains of attention and executive abilities, psychomotor function, visuospatial skills, memory, general intellectual abilities and mood, as defined in Sullivan et al.[31,33]. This battery includes tests shown to have high specificity and sensitivity for detecting changes in neuropsychological functions between veterans with and without GWI[21]. Cross-sectional and longitudinal correlation analyses with neuroimaging and neuropsychological outcomes on measures of attention and executive functioning (COWAT, D-KEFS Color Word Interference, Trail Making B and CPT-III) are planned. Additional analyses will compare learning slopes on the CVLT verbal memory task in addition to fine motor changes over time on the Finger Tap Test and Grooved Pegboard. We hypothesize that the cross-sectional and longitudinal analyses will show more significant deficits in veterans with GWI due to brain function alterations.

GWIC and BBRAIN Blood biomarkers: Use of biorepository samples and Sample Quality Control Assessment.

EM Papper biorepository of Excellence: The EM Papper Clinical Immunology Laboratory at Nova Southeastern University (NSU) has been shown to be have a profile of excellence in GWI, ME/CFS and HIV research for over 30 years. This laboratory is also an official IMPACT/ACTG/WIHS Laboratory for processing of specimens and testing of samples. The EM Papper lab has supported multiple NIH and VA funded research groups, with a biorepository, for more than 20 years. The laboratory has full generator back up and supported storage, with sufficient - 20 and -80 degree C freezers, and liquid nitrogen storage and IATA shipping certification for all staff members. It is a fully licensed clinical reference laboratory by CLIA, the State of Florida, and COLA. Laboratory members have sufficient experience and expertise in the proposed methods and planned lab testing. All facilities have temperature monitoring and emergency backup, and are maintained daily. The GWIC and BBRAIN blood biorepositories are located at NSU. The director of the lab is Dr. Klimas and Dr. Fletcher and Co-Director is Dr. Abreu. They have worked extensively with Dr. Sullivan as the primary biorepository site for the Boston GWIC and created well-functioning SOPs for biospecimen collection, shipment, storage and sample sharing among GWI researchers. These SOPs are utilized in the BBRAIN repository. Study samples will be run at NSU from the BBRAIN repository and sample quality be assessed before analyses.

Sample Quality Control and Assessment – The NSU laboratory participates in quarterly assessments of its ability to process, store, and ship cryopreserved Peripheral Blood Mononuclear Cells (PBMCs), by the Duke University Immunology Quality Assessment. This initiative has been supported by the NIH to increase the quality of biorepositories. The EM Papper Laboratory has participated and received the longest standing level of excellence in the USA. Quality Assurance and Assessment protocols take place before, during and after samples are isolated from primary tubes and placed into the biorepository. Daily logs of freezer temperatures are maintained.

Plasma Cytokine Analysis. An aliquot of plasma will be used to measure cytokines in BBRAIN (18 cytokine multiplex panel), The pro inflammatory cytokine cascade including IL1α and IL1β, and soluble receptors for TNFr1, TNFr2, IL1r , as well as Th1, Th2,Th17 and anti-inflammatory cytokines will be measured with a multiplex chemo- luminescent assay (Quansys). Calcium levels will also be shared through BBRAIN.

Glutamate and phosphate analysis. The NSU EM Papper laboratory will perform the glutamate and phosphate analyses for Time 2 data sets. The T1 data will be obtained from GWIC samples that will already have been analyzed for these markers in the same laboratory using the same methods. Time 2 analyses for this study will be performed using a fluorimetric glutamate assay kit for the measurement of as little as 1μM glutamate (glutamic acid) in plasma. The phosphate assay is a colorimetric assay with a sensitivity of 1μM. Serum samples are run in duplicate for assays and data is analyzed using a five-parameter logistic regression.

Data analyses: Analysis for Aim 1, refining the pathobiology of WM microstructural pathways and crossing fibers in GWI, will focus on the comparison of GWI cases and healthy veteran controls on MRI imaging parameters through linear regression models that allow for the control of relevant covariates and potential confounding factors such as age and sex. We will control for the multiple comparisons issue of examining a large number of parameters through the false discovery rate using the method of Benjamini, Hochberg, and Yekutieli. Aim 2 will focus on differential longitudinal change in brain volumetric, microstructural and CBF parameters in GWI cases vs. healthy veteran controls using mixed effects linear regression models for longitudinal data. The models will include a random subject effect to account for

the correlation between repeated measures for each subject, and terms for GWI status and time, as well as relevant covariates. Differential longitudinal patterns in GWI vs. controls subjects will be modeled by the interaction between GWI status and time. We will control for the multiple comparisons issue through the false discover rate.

Sample size and Power considerations: For Aim 1, with 50 GWI cases and 50 health controls, the proposed study has 80% power of detecting differences in MRI parameters between cases and controls corresponding to a standardized effect size (Cohen's d) of 0.75, corresponding to a medium-to-large effect (using a two-tailed alpha of 0.005 to account for the multiple comparisons issue). For Aim 2, approximating power of the mixed effects regression models through an analysis on difference scores, and assuming a moderate to strong correlation (r=0.5 to 0.75) between repeated measures of MRI parameters, the proposed study has 80% power of detecting a difference in longitudinal change corresponding to a standardized effect size (Cohen's d based on mean change divided by the baseline standard deviation) between 0.4 and 0.75, representing medium to large effects.

Impact: Determining the association between CBF patterns, BBB permeability and WM microstructural integrity and WHM will identify better understanding of the exact pathobiology of GWI. This study will have a tremendous impact on GW veterans because the results can then be utilized to assess disease severity and progression hopefully in time before clinically significant cerebral small vessel disease results in at-risk veterans. These markers can also be used for targeted treatment strategies and to quantitatively measure treatment trial efficacy on these outcomes.

Specific Aims:

Aim 1: To compare, validate and further refine the pathobiology of WM microstructural pathways and crossing fibers in GWI.
Aim 2: To compare longitudinal patterns of brain volumetric, microstructural and CBF differences in 50 GWI cases and 50 healthy control veterans compared with neuropsychological and blood biomarkers.
Aim 3: To perform machine learning advanced analytic data reduction analyses on GWIC and BBRAIN imaging, neuropsychological and blood biomarkers to predict GWI case status and symptom severity over time.

4. KEY RESEARCH ACCOMPLISHMENTS

The approved statement of work for the entire study period is below:

STATEMENT OF WORK

Table 1. Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers of White Matter Integrity and Hemodynamic Response

Tasks	Timeline
Task 1. Obtain necessary authorization prior to initiation of human subjects	**Months**
1a. Obtain Institutional Review Board (IRB) approval for research sites at, Boston University Medical Campus (BUMC), Nova University (NSU) and Baylor Medical College for protocols	1-4
1b. Obtain DOD Human subjects Research Protections Office (HRPO) approvals	5-7

1c. Complete hiring of necessary staff and ensure all mandatory IRB research related trainings are completed by all staff members	1-8
Task 2. Preparation and Training for Clinical Study Procedures	**Months**
2a. Obtain Time 1 GWIC cognitive, MRI neuroimaging and blood biomarkers data for longitudinal analyses.	1-2
2b. Develop manuals for structural MRI, DTI, fMRI and pCASL imaging and clinical examination protocols at Boston University and perfusion study manuals at Baylor University	1-6
2c. Train researchers and staff on neuroimaging and clinical examination protocols and quality control measures.	6-9
Task 3. Screening, recruitment and longitudinal assessment of GWIC/BBRAIN Gulf War veterans	**Months**
3a. Obtain informed consent from potentially eligible GW veterans	9-36
3b. Assess 100 (50 cases/50 controls) GWIC/BBRAIN veterans and obtain demographics and clinical examination (BP, HR, pulse) for planned longitudinal analyses at Boston University.	9-36
3c. Perform structural, functional and cerebral blood flow MRI imaging from 100 (50 cases/50 controls) Gulf War veterans for longitudinal BBRAIN call back study at Boston University.	9-36
3d. Perform perfusion MRI validation pilot study with 5 GW veterans at two time points at Baylor Medical College.	9-24
3e. Obtain BBRAIN Time 2 cognitive, demographic and blood biomarker data for planned longitudinal analyses.	9-36
Task 4. Perform post-processing of MRI, DTI, fMRI, pCASL, perfusion image data and analyze stored BBRAIN blood samples	**Months**
4a. Post-process MRI, DTI, fMRI, pCASL, perfusion neuroimaging data for data analysis at Boston University and Baylor Medical College.	11-34
4b. Merge BBRAIN neuropsychological test data, demographics and blood study outcomes with MRI imaging and other study data for analysis at Boston University.	11-34
4c. Perform analysis of stored BBRAIN blood samples for phosphate and glutamate levels at Nova Southeastern University where BBRAIN samples are stored.	11-34
4d. Obtain phosphate and glutamate results and merge with brain imaging and cognitive data for planned analyses.	11-34
Task 5. Merge Data and Perform Interim Data analyses	**Months**
5a. Data merging of BBRAIN longitudinal data including questionnaires, cognitive evaluations and clinical evaluations with current brain imaging data.	11-24
5b. Interim statistical analyses of data obtained from brain imaging outcomes, cognitive evaluations and blood markers will be performed periodically.	11-24
5c. Annual reports of progress will be written.	12-24
Task 6. Perform Final Data Analysis, Prepare Manuscripts for Publication and share brain imaging data with BBRAIN	**Months**
6a. Perform cross-sectional and longitudinal analyses comparing brain imaging outcomes over time and with demographic and cognitive outcomes and with blood markers including phosphate, glutamate and cytokine/chemokines.	24-34
6b. Perform perfusion pilot study data analysis to validate results with pCASL and fMRI cerebral blood flow outcomes.	24-26
6c. Perform final machine learning algorithm and analyses	32-36
6d. Write final study report	24-26

6e. Present findings at scientific meetings and prepare manuscripts for submission for perfusion validation, cross-sectional and longitudinal and machine learning studies.	24-36

Accomplishment under these goals.

TASK 1. OBTAIN NECESSARY AUTHORIZATION PRIOR TO INITIATION OF HUMAN SUBJECTS

Task 1a. Obtain Institutional Review Board (IRB) approval for research sites at, Boston University Medical Campus (BUMC), Nova University (NSU) and Baylor Medical College for protocols

Institutional Review Board (IRB) approvals have been obtained at Boston University Medical Campus and exempt status will be obtained at NSU. IRB protocol has been submitted at UT-Houston and Baylor Medical college and should be approved soon.

Task 1b. Obtain DOD Human subjects Research Protections Office (HRPO) approvals

The HRPO protocol has been submitted for the BU site and after discussions with HRPO, BU will be the IRB of record for this study. Agreements are underway now to approve this at the other study sites. Once the agreements are in place, the submission will be further reviewed by HRPO for approval.

Task 1c. Complete hiring of necessary staff and ensure all mandatory IRB research related trainings are completed by all staff members

All necessary staff have been hired and all mandatory IRB training have been completed by all staff members.

TASK 2. PREPARATION AND TRAINING FOR CLINICAL STUDY PROCEDURES

Task 2a. Obtain Time 1 GWIC cognitive, MRI neuroimaging and blood biomarkers data for longitudinal analyses.

GWIC received a no-cost extension in order to extend recruitment to try to see more Gulf War veteran controls. Once we have recruited the final sample, we will then obtain all the GWIC cognitive, MRI neuroimaging, and blood biomarkers data for longitudinal analyses. In the meantime, neuroimaging papers are being completed on the Time 1 dataset from GWIC.

Task 2b. Develop manuals for structural MRI, DTI, fMRI and pCASL imaging and clinical examination protocols at Boston University and perfusion study manuals at Baylor University

The imaging protocol has been finalized for this study and subject recruitment approval has been requested to release the pandemic related hold on in-person studies at BUMC.

Task 2c. Train researchers and staff on neuroimaging and clinical examination protocols and quality control measures.

A detailed in-person training session for the parent study BBRAIN was held in October 2019 at the Boston University Coordinating Center for all clinical research personnel who will be working directly

with study participants to ensure adequate quality control of test administration and interview procedures among the study sites. All neuropsychological testing materials and survey instruments have been purchased. Quality control measures will continue to be instituted and monitored by experienced Administrative Core investigators including Dr. Toomey as the clinical studies proceed to ensure good inter-rater reliability and to reduce tester drift. All personnel were trained on the neuroimaging procedures for this study.

TASK 3. SCREENING, RECRUITMENT AND LONGITUDINAL ASSESSMENT OF GWIC/BBRAIN GULF WAR VETERANS

Task 3a. Obtain informed consent from potentially eligible GW veterans

To date, two Gulf War veterans have been assessed for the parent BBRAIN study. However, these veterans were not eligible for this study's MRI component. Because of COVID-19 recruitment has been staggered. However, we have received approval for research resumption again for the parent BBRAIN study. We have sent in the required paper work for the BBRAIN MRI study, and will begin recruitment once approved for in-person visits for the imaging studies.

Task 3b. Assess 100 (50 cases/50 controls) GWIC/BBRAIN veterans and obtain demographics and clinical examination (BP, HR, pulse) for planned longitudinal analyses at Boston University.

Once research resumption approvals are granted, we will begin in-person subject recruitment visits right away. We hope this will be in the very near future.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

- **Research Staff Training:** As a large academic community, Boston University also provides opportunities for students to get real world experience. The parent BBRAIN study can tap into this talent pool by taking practicum and intern students in the fields of health communication and promotion, who as part of an academic program, will create a media and outreach program for the network. Doctoral students in related fields can also be brought on to aid in this project. Finally, as has been exhibited with the GWIC, the network will be financially continued through spin-off grants in GWI and similar fields through DOD, VA and NIH and by providing the tools for talented young researchers to aid us in tackling and solving the problem of GWI. For example, in summer of 2019 we had a student from Middlebury College, VT working with us a summer intern who helped develop the BBRAIN website. This study will be listed as a follow-up study to the BBRAIN study on the BBRAIN website and social media pages by our students and staff members.

- **Participation in conferences, workshops, and seminars**: BBRAIN MRI researchers will attend conferences, workshops and seminars to present research findings.
    - On October 3rd 2019, Drs. Sullivan and Klimas presented a talk titled, 'Military Veteran Biorepositories' where they explained available biorepositories for military research and discussed plans for BBRAIN sharing of samples for military veteran studies as part of the National Academy of Sciences (NAS) workshop on Gulf War Respiratory Health Workshop on Military Burn Pits in Washington, DC. This Committee will determine next steps for burn pit studies for Iraq war veterans and for GW veterans with respiratory problems.
    - On October 11-13th 2019, Drs. Sullivan and Klimas were invited keynote speakers at the American Academy of Environmental Medicine (AAEM) annual meeting in

Louisville, KY. Dr. Sullivan's talk was titled 'Neuropathology and Toxicology of Gulf War Illness and Fatigue-related Disorders' and Dr. Klimas' presentation was titled, 'Can We "Reboot" Human Homeostasis to Cure Chronic Illness? What We Are Learning from Gulf War Illness and ME.'

- On February 28th, 2020, Drs. Sullivan and Klimas were invited key note speakers and presented a talk titled, "Moving Knowledge to Treatment" at the State of the Science: Gulf War Illness conference in Ft. Lauderdale, FL.
- In August 2020, Dr. Sullivan presented an overview of the BBRAIN and follow-up studies including this study to the DOD/VA 30th Anniversary of Operation Desert Storm Gulf War Illness virtual conference.

- **How results will be disseminated to communities of interest.**
  - Research staff will use some of the outreach structure already in place from the Boston GWI consortium. The Facebook page has over 2,500 followers, many of whom regularly interact with and share page content. This allows researchers to freely spread information among the already existing GWI network on social media. GWIC also has a Twitter account and study website that will be used to disseminate information to the community and refer to the parent BBRAIN study social media sites. Boston University also has a very proactive media team that produces text and video news stories that get widely shared. Research staff will also be aided by the Veteran Working Group in getting news out about the parent BBRAIN study to fellow veterans and their communities. As the research team has done for GWIC and BBRAIN, they will write professional publications that state what samples are available and who to contact for this BBRAIN MRI study.

- **Plan to do during the next reporting period to accomplish the goals:** In the next reporting period, we plan to:
  - Receive research resumption approvals from BUMC to see study participants in person.
  - Begin subject recruitment. Process, store and manage samples for a total of 100 participants. We also plan to merge data and perform interim data analyses.
  - We believe that our media stories and recruiting participants from our ongoing consortium studies will catch us up on subject recruitment by the end of year 2.

5. IMPACT

- **Impact on the development of the principal discipline(s) of the project:**
  - *"Nothing to Report."*

- **Impact on other disciplines:**
  - We are working to develop new brain imaging and machine learning analysis as diagnostic markers for GWI. We also hope to use these markers for treatment development and efficacy analyses. The first publication has now been completed on this work at https://www.mdpi.com/2076-3425/10/11/884. This technology could also then be utilized in other disorders including TBI and chronic multisymptom disorders including ME/CFS.

- **Impact on technology transfer:** *If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *Nothing to Report.*

- **Impact on society beyond science and technology:**
  - *Nothing to Report.*

6. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
  - *Nothing to Report*

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - We have been on hold to start subject recruitment due to the COVID 19 pandemic which has caused a delay in this study. We hope to be able to get subject recruitment started soon to catch up with our recruitment plans.

- **Changes that had a significant impact on expenditures**
  - *Nothing to Report*

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - *Nothing to Report*

7. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  - **Journal publications.**
    - We have published one recent article related to machine learning and diagnostic markers using brain imaging in veterans with GWI.
  - Neuroimaging Markers for Studying Gulf-War Illness: Single-Subject Level Analytical Method Based on Machine Learning Yi Guan 1,† , Chia-Hsin Cheng 1, Weifan Chen, Yingqi Zhang, Sophia Koo, Maxine Krengel, Patricia Janulewicz, Rosemary Toomey, Ehwa Yang, Rafeeque Bhadelia, Lea Steele, Jae-Hun Kim, Kimberly Sullivan and Bang-Bon Koo 1. Brain Sci. 2020, 10, 884; doi:10.3390/brainsci10110884 Link: https://doi.org/10.3390/brainsci10110884
  - **Books or other non-periodical, one-time publications.**
    - *Nothing to Report.*
  - **Other publications, conference papers, and presentations.**
    - *Nothing to Report.*
  - **Website(s) or other Internet site(s)**
    - http://sites.bu.edu/bbrain/

- https://wwwapp.bumc.bu.edu/BEDAC_BBrainRetro

- **Technologies or techniques**
  - *Nothing to Report.*
- **Inventions, patent applications, and/or licenses**
  - *Nothing to Report.*
- **Other Products**
  - *Nothing to Report.*

8. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **Individuals that have worked on the project:**

Name: Kimberly Sullivan	Project Role:	*PI of Biorepository Network and Boston Site*
	Researcher Indentifier (e.g. ORCID ID):	*https://orcid.org/0000-0001-7940-6123*
	Nearest person month worked:	
	Contribution to Project:	Oversee coordination for data collection of brain imaging and sharing of blood and cognitive data for analysis.
	Funding Support:	
Name: Nancy Klimas	Project Role	
	Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0003-1459-3268*
	Nearest person month worked:	
	Contribution to Project:	Will oversee the analysis of blood biomarkers
	Funding Support:	
Name: Deborah Little	Project Role:	*Co-investigator*
	Researcher Identifier: (e.g. ORCID ID):	https://orcid.org/0000-0003-4498-4649
	Nearest person month worked:	
	Contribution to Project:	Responsible data collection, analysis, integration with ASL and evaluation of cortical volumes and thickness for perfusion validation
Name: Emily Sisson	Project Role	*Data Manager/Analyst*

	Researcher Identifier: (e.g. ORCID ID):	
	Nearest person month worked	
	Contribution to Project:	Data Management Specialist
Name: Ronald Killiany	Project Role	*Co-Investigator*
	Researcher Identifier: (e.g. ORCID ID):	*https://orcid.org/0000-0003-4740-2181*
	Nearest person month worked	
	Contribution to Project	Imaging Director; Oversee MRI Images
Name: Bang-Bon Koo	Project Role	*Co-Investigator*
	Researcher Identifier (e.g., ORCID ID):	*https://orcid.org/0000-0001-7423-5572*
	Nearest person month worked	
	Contribution to Project	Machine Learning and Imaging Expert; Conduct MRI imaging
Name: Maxine Krengel	Project Role	*Co-Investigator*
	Researcher Identifier (e.g., ORCID ID):	*https://orcid.org/0000-0001-7632-590X*
	Nearest person month worked	
	Project Role	Neuropsychologist; Neurobehavioral Core Director
Name: Timothy Heeren	Project Role	*Co-Investigator*
	Researcher Identifier (e.g., ORCID ID):	*https://orcid.org/0000-0001-5643-3559*
	Nearest person month worked	
	Project Role	Senior Biostatistician

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - *Nothing to report.*
- **What other organizations were involved as partner?**
  - **Organization Name:** Boston University Medical Campus
  - **Location of Organization:** Boston, Massachusetts
  - **Partner's contribution to the project**
    - **Financial support:** *Nothing to Report*
    - **In-kind support:** *Nothing to Report*
    - **Facilities:** *Nothing to Report*

        - **Collaboration:** Drs. Bang Bon Koo, Ronald Killiany and Maxine Krengel, BBRAIN MRI Co-Investigators affiliated with Boston University Medical Campus. Dr. Bang-Bon Koo, a clinical faculty member in the Anatomy and Neurobiology department at BUMC will assist with machine-learning analyses in GWI biomarker and diagnostic analyses. Dr. Ronald Killiany, director of the BUMC Center for Biomedical Imaging will serve as the Imaging Core Director and will oversee compilation of imaging data and data mining/post-processing samples. Dr. Bang-Bon Koo, a clinical faculty member in the Anatomy and Neurobiology department at BUMC will also be available to assist with machine-learning analyses in GWI biomarker and diagnostic analyses. Dr. Deborah Little is an imaging expert at UT-Houston and will assist with image analyses. Dr. Nancy Klimas will assist with laboratory analyses of blood biomarkers glutamate and phosphate.
        - **Personnel exchanges:** *Nothing to Report*
        - **Other:** *Nothing to Report*
    - **Organization Name:** NOVA Southeastern University
    - **Location of Organization:** Fort Lauderdale, Florida
    - **Partner's contribution to the project:** This lab will run the blood biomarkers in the study
        - **Financial support:**
        - **In-kind support:**
        - **Facilities:**
        - **Collaboration:**
        - **Personnel exchanges:**
        - **Other:**
    - **Organization Name:** Baylor Medical College/ UT-Houston
    - **Location of Organization:** Houston, Texas
    - **Partner's contribution to the project:** The lab will assist with image analyses for the study.
        - **Financial support:**
        - **In-kind support:**
        - **Facilities:**
        - **Collaboration:**
        - **Personnel exchanges:**
        - **Other:**

9. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

  Nothing to report

- **QUAD CHARTS:**

10. APPENDICES:

Appendix:

Please see attached publication:

Yi Guan, Chia-Hsin Cheng, Weifan Chen, Yingqi Zhang, Sophia Koo, Maxine Krengel, Patricia Janulewicz, Rosemary Toomey, Ehwa Yang, Rafeeque Bhadelia, Lea Steele, Jae-Hun Kim, Kimberly Sullivan and Bang-Bon Koo. Neuroimaging Markers for Studying Gulf-War Illness: Single-Subject Level Analytical Method Based on Machine Learning Brain Sci. 2020, 10, 884; doi:10.3390/brainsci10110884
Link: https://doi.org/10.3390/brainsci10110884

brain sciences

MDPI

Article

Neuroimaging Markers for Studying Gulf-War Illness: Single-Subject Level Analytical Method Based on Machine Learning

Yi Guan [1,†], Chia-Hsin Cheng [1,†], Weifan Chen [1], Yingqi Zhang [1], Sophia Koo [1], Maxine Krengel [1], Patricia Janulewicz [2], Rosemary Toomey [1], Ehwa Yang [3], Rafeeque Bhadelia [4], Lea Steele [5], Jae-Hun Kim [3], Kimberly Sullivan [2,*] and Bang-Bon Koo [1,*]

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Abstract: Gulf War illness (GWI) refers to the multitude of chronic health symptoms, spanning from fatigue, musculoskeletal pain, and neurological complaints to respiratory, gastrointestinal, and dermatologic symptoms experienced by about 250,000 GW veterans who served in the 1991 Gulf War (GW). Longitudinal studies showed that the severity of these symptoms often remain unchanged even years after the GW, and these veterans with GWI continue to have poorer general health and increased chronic medical conditions than their non-deployed counterparts. For better management and treatment of this condition, there is an urgent need for developing objective biomarkers that can help with simple and accurate diagnosis of GWI. In this study, we applied multiple neuroimaging techniques, including T1-weighted magnetic resonance imaging (T1W-MRI), diffusion tensor imaging (DTI), and novel neurite density imaging (NDI) to perform both a group-level statistical comparison and a single-subject level machine learning (ML) analysis to identify diagnostic imaging features of GWI. Our results supported NDI as the most sensitive in defining GWI characteristics. In particular, our classifier trained with white matter NDI features achieved an accuracy of 90% and F-score of 0.941 for classifying GWI cases from controls after the cross-validation. These results are consistent with our previous study which suggests that NDI measures are sensitive to the microstructural and macrostructural changes in the brain of veterans with GWI, which can be valuable for designing better diagnosis method and treatment efficacy studies.

Keywords: Gulf War illness; MRI; objective biomarker; machine learning; Kansas case criteria; diffusion; grey matter; neurite density imaging

1. Introduction

Gulf War illness (GWI) refers to the variety of chronic symptoms experienced by about 250,000 United States veterans who served in the 1991 Gulf War (GW) [1]. According to the Kansas

case criteria, symptoms of GWI fall into six categories: fatigue (fatigue and sleep problems), pain (joint and muscle), neurological (cognitive, mood, headache, and dizziness), respiratory (persistent cough and wheezing), gastrointestinal (diarrhea and nausea), and skin (rashes and other) problems. Exposure to neurotoxicant chemicals (organophosphate pesticides and sarin) during the war and other central nervous system (CNS) damage, such as mild traumatic brain injury (mTBI), are thought to have caused an innate immune over-response in the CNS, resulting in the development of these chronic GWI symptoms [2–7]). In order to meet the Kansas criteria for GWI, veterans must display chronic symptoms in at least three of the six categories, without presenting concurrent psychiatric and medical disorders [8]. However, accurate diagnoses of GWI remained challenging due to the heterogeneous clinical presentation of this condition, as well as the level of subjectivity associated with self-reported symptoms and neurotoxicant exposure history [8–10]. To improve management and treatment of GWI, there is an urgent need for defining sensitive and objective biomarkers of the disorder.

Previous neuroimaging studies demonstrated distinct changes within brains of veterans with GWI, which may underlie physiological symptoms. For example, T1W-MRI studies showed that GW veterans with exposure to the neurotoxicant chemical sarin exhibit reduced gray matter (GM) and white matter (WM) volumes, as well as reductions in hippocampal subfield volumes when compared to non-exposed veterans [11,12]. More recent studies using diffusion tensor imaging (DTI) have shown greater hippocampal mean diffusivity (MD) and increased axial diffusivity (AD) in the WM of sarin and cyclosarin exposed GW veterans, which are correlated to fatigue, pain, or hyperalgesia, and may serve as a potential biomarker for GWI [13–15]. We have previously applied a novel MRI diffusion processing method, neurite density imaging (NDI), on high-order diffusion MRI to demonstrate that the NDI measure scan successfully identify and validate different levels of neurological abnormalities in veterans with GWI from the Boston Gulf War Illness Consortium cohort [16].

ML algorithms have been applied to study a wide range of neurological disorders, including Alzheimer's disease, Parkinson's disease, and traumatic brain injury [17,18]. These studies have reported promising results for identifying diagnostic biomarkers [19,20]. The ML approach have strengths on exploiting features from different domains (i.e., neuropsychological, genetic and neuroimaging) and providing further insights on the potential interactions between different markers for classifying illness [21]. For the current study, we aimed to expand our previous work (on NDI) to cross-compare different types of neuroimaging markers (T1W-MRI, DTI and NDI) to determine whether these measures are useful for single subject-level classification of GWI cases vs. controls. Specifically, we incorporated the machine learning (ML) framework to search out key imaging features valuable for defining GWI. Computerized models were then trained based on the selected features and tested for classifying veterans with GWI.

2. Methods

2.1. Participants

In this study, we included brain imaging data of 119 GW veterans from Boston University Gulf War Illness Consortium (GWIC) (Table 1). GWIC is a multi-site study designed to identify the etiology and potential biomarkers of GWI. The inclusion criterion was deployment to the GW between August 1990 and July 1991. The exclusion criteria included having a diagnosis of chronic medical illnesses that could otherwise account for the symptoms experienced by GW veterans, including autoimmune, CNS, or major psychiatric disorders that could affect the brain and immune functions (e.g., epilepsy, stroke, severe head injury, etc.). Each participant completed an assessment protocol of health surveys, a neuropsychological test battery, brain imaging, and collection of blood and saliva samples [2]. In this study, we utilized brain imaging outcomes to study GWI. All participants provided written informed consent to participate in the study. This study was reviewed and approved by the Boston University institutional review board.

Table 1. Subject Characteristics.

BU Subjects	GW Control	GWI Case
N	21	98
Age (years)	54.06	52.46
Gender (F/M)	3/18	20/78

Gulf War Illness Criteria and Symptom Surveys

GWI case status was defined from the Kansas GWI case definition, which requires multiple or moderate-to-severe chronic symptoms in at least three of six statistically defined symptom domains: fatigue/sleep problems, somatic pain, neurological cognitive/mood symptoms, gastrointestinal symptoms, respiratory symptoms, and skin abnormalities [8]. GWIC participants not meeting Kansas GWI or exclusionary criteria were considered controls. Veterans were excluded from being considered GWI cases, for purposes of the research study, if they reported being diagnosed by a physician with medical or psychiatric conditions that would account for their symptoms or interfere with their ability to report their symptoms. GWIC subjects were administered a general demographic information and medical conditions questionnaire and the Kansas Gulf War and health questionnaire for assessing symptoms [8,10]. Additional validated health symptom surveys were completed by study participants and included the multidimensional fatigue inventory (MFI-20), McGill pain inventory and the Pittsburgh sleep quality index (PSQI) where higher scores suggested worse conditions [22–24].

2.2. Image Acquisition

All veterans were scanned on an Achieva 3T whole-body MRI scanner (Philips Healthcare, Best, The Netherlands) at the Center of Biomedical Imaging, Boston University school of Medicine. T1W-MRI were obtained using an MPRAGE sequence developed by the Alzheimer's disease neuroimaging initiative (ADNI) (Repetition time (TR) = 6.8 ms, Echo time (TE) = 3.1 ms, flip angle = 9°, slice thickness = 1.2 mm, 170 slices, Field of view (FOV) = 250 mm, matrix = 256 × 256) (accessible from http://adni.loni.usc.edu/). Diffusion MRI data were obtained using 124 gradient directions utilizing parallel imaging on a 16-channel parallel head coil (70 slices, TR = 13,214 ms, TE = 55 ms, with a matrix size of 128 × 128 yielding a resolution of 2.0 × 2.0 × 2.0 mm^3, no slice gap). Multi-shell diffusion encodings with b-values 1000, 2000 and 3000 s/mm^2 were acquired with a single-shot echo planar imaging (EPI) sequence, and 6 b = 0 s/mm^2 field maps were collected in addition to distortion corrections built into the scanner.

2.3. Image Processing and Anatomical Defining

Structural T1W-MRI scans were analyzed with the Freesurfer package (version 6.0) to generate anatomical regions of interest (ROI) for assessing GM morphometric measures, and to provide GM anatomical co-registration references for diffusion images [25]. A total of 78 ROIs defined in the average template space were co-registered to each subject's cortical surface by applying nonlinear co-registration parameters. All results were visually inspected for artifacts or incomplete segmentation. Fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) maps were created using tract-based spatial statistics (TBSS), part of FSL package that projects all subjects' diffusion tensor imaging (DTI) data onto a mean tract skeleton [26]. A total of 20 major WM tracts were defined using the Johns Hopkins University (JHU) white-matter tractography atlas provided in the FSL package, the same template was also used for special normalization and linear co-registration of diffusion MRIs [27,28].

2.4. High-Order Diffusion Processing

Microstructural diffusion measures were reconstructed from multi-shell diffusion MRI images containing 3 b-value encodings using the NDI model [16]. Two parameters, neurite density (ND)

index and orientation dispersion (OD) index were extracted from the NDI model. In brief, ND is a fraction of tissue composed of neurites which include axons and dendrites, and OD provides the spatial configuration of the neurite structures based on the composite pattern of intra- and extracellular diffusivity [29]. For WM NDI measures, all subjects' NDI data were registered to a common space based on nonlinear transformation and projected to the WM tract skeleton. Next the major WM tract ROIs were then applied to the skeletonized WM NDI maps to extract ROI-wise NDI measures [26]. For the GM diffusivity assessment, diffusion modeling parameters were determined by voxel wise iterative parameter selection method. We used the maximum likelihood estimation of model fitting error to define the optimal intrinsic free diffusivity parameters [30]. The optimal parameters were used to reconstruct the GM NDI maps and then merged into the 78 GM ROIs to extract ROI-wise NDI measures [30,31].

2.5. T1-Weighted MRI Measures

From the Freesurfer cortical reconstruction process of T1W-MRI, we extracted six measures per subject, including cortical thickness, cortical surface area, cortical volume (cVolume), subcortical GM volume (scVolume), WM volume, curvature (curv). Specifically, cortical thickness, surface area, volume, and curvature are extracted from 62 ROIs based on Desikan–Killiany–Tourville (DKT) atlas, while subcortical ROIs are defined by Freesurfer built-in atlas [31,32].

2.6. Statistical Analysis

From the data processing steps, we generated in total 14 types of imaging measures: 4 NDI, 4 DTI, and 6 T1-weighted morphometric measures. For each type of imaging measure, we conducted statistical comparisons of GWI cases vs. controls using linear regression models adjusting for age and sex, and then corrected for multiple comparison using false discovery rate (FDR) [33]. We reported t-values and FDR-corrected p-values (FDR-p), significant features are defined as FDR-$p < 0.05$.

2.7. Machine Learning Classification

Imaging measures described in the previous sections are used as pre-defined features for training ML classification models. Age- and sex-related confounds were removed from the raw data before training the model. This step is achieved by estimating the effects of age and sex on imaging measures using a linear regression model that is similar to a method applied in an early study [19]. For building the classifier for each imaging measure we adapted a reinforcement learning algorithm with artificial bee colony algorithm for feature selection (BSO: bee swarm optimization), and the K nearest neighbors (KNN) algorithm for classification training and performance evaluation [34,35].

2.7.1. Feature Space Selection and Classifier Training

As mentioned previously, some specific neuroimaging markers (i.e., NDI measures) may be more sensitive for detecting the subtle neurological changes occurring in GWI cases [16]. For training the classifiers, each type of imaging measures (i.e., measurement domains) serves as prior information that will allow us to set up specific feature space for potentially better ML outcomes. Within each feature space, reinforcement learning-based BSO (QBSO) was used to perform iterative search of the subset of features that provides the best classification performance on the training dataset (more details described in QBSO Tuning). Through QBSO, a final subset of features (final solution) was selected to build a final classifier. Final classifiers trained on each feature space were- then tested on the validation dataset (see more details in Ensemble Approach).

QBSO Tuning

This feature selection concept combines the BSO and reinforcement learning (specifically Q-learning) to upgrade simple local search to a more adaptive and efficient search for the final

solution [34,35]. Previous study has shown that this hybrid method outperforms other well-known ML algorithms for feature selection [35]. More specifically, the BSO method mimics the foraging behavior of natural bees by performing iterative local search for an optimized solution [36].

From the predefined feature space explained earlier, the initial solution is randomly generated. Then, BSO randomly modifies the initial solution to multiple different secondary solutions, where each will be assigned to a bee (an agent) to perform local search to find local optimum (based on k-fold cross-validation accuracy). In this local search stage, each bee refers to a series of experiments obtained in previous steps to make a decision to do further search in the current search pace, and this local search will continue until no further improvement of accuracy occurs. When the bee reaches this point, each bee's search history is shared to other bees and used for the diversification of searching process.

In the diversification process, the most distant solution will be selected based on the shared information. During this process, the role of reinforcement learning is to allow the agent learn through an interactive environment by trial and error. As the result, the QBSO method will search for a solution (i.e., resulting feature list) that maximizes the reward through multiple iterations. In each iteration, KNN runs on the candidate features (one of the secondary solutions) selected from the bee and tested for 5 iterations of 5-fold cross-validation on the training dataset. We used an average accuracy measure from the 5-fold cross-validation for estimating the reward. Finally, the search process will terminate based on the pre-defined parameters. To set up the optimal parameters, we used a grid-search strategy that is empirically searching the parameters resulting in the highest classification accuracy for the training dataset. The final parameters used in this experiment are listed as follows: flip: 20, max. chance: 9, nBees: 30.

Ensemble Approach

Per each feature space (i.e., one type of imaging measure), QBSO produces a subset of final features that provides the highest average accuracy from the iterative search. QBSO is repeated 5 times in total to generate 5 final solution candidates for a single training dataset. Per each solution, we built 3 different classifiers- KNN, support vector machine, and random forest classifiers. The training dataset was further split into 2 parts (i.e., training and testing) and used to train each classifier. Then the weighted majority voting was used to ensemble those 15 classifiers (i.e., 3 classifiers from each solution) to make a final prediction on the validation dataset. The following weight function was used: $Wi = Pi/(1 - Pi)$, Pi: performance of i-th classifier, i = [1:15].

2.7.2. Comparing Classification with Different Imaging Measures

As mentioned previously, each type of imaging measures was used to set up distinct candidate feature space for training the classifiers. The resulting 14 different classifiers (4 NDI, 4 DTI, and 6 T1W-MRI morphometric measures) were evaluated based on their classification performances. For the benchmark testing, the entire dataset was initially divided into a training dataset and a validation dataset based on a 5-fold partitioning. We took one fold as a validation dataset and used the remaining 4-fold data for performing the QBSO training framework (Section 2.7.1). This process was repeated 5 times as training/validation datasets rotate among the 5 folds (by taking each fold as the validation dataset in each iteration). For the classification performance comparison, we reported performance measures (averaged from 5 iterations after validation) of accuracy, sensitivity, specificity, and F-score. We included F-score as a more representative performance measure for the imbalanced case and control groups [37]. In addition to the average accuracy, we included the standard deviation (SD) of accuracy, as an estimate of variations between iterations, and the highest accuracy value for the top three classifiers.

3. Results

3.1. Group-Level Statistical Comparison and Key Imaging Features

Statistical analysis of NDI measures showed significant differences between GWI cases and controls in both WM tracts and GM ROIs (FDR-$p < 0.05$) (Figure 1). The full result can be found in Table S1. All major WM tracts showed significant decreases in ND and OD for GWI cases compared to controls (Figure 1A). The greatest significant group differences between GWI cases and controls were seen in the bilateral corticospinal tract (CST, t = −3.119 FDR-$p = 0.017$ (left), t = −3.129, FDR-$p = 0.017$ (right)) and the bilateral anterior thalamic radiations (ATR, t = −2.891, FDR-$p = 0.017$ (left), t = −2.808, FDR-$p = 0.017$ (right)) for WM ND, and in the bilateral cingulum cingulate gyrus bundle (CCG, t = −4.041 FDR-$p = 0.002$ (left), t = −3.384, FDR-$p = 0.007$) for WM OD. Both ND and OD showed decreased patterns (FDR-$p < 0.05$) for most GM ROIs as well (Figure 1B). The greatest significant group differences between GWI cases and controls were seen in the left isthmus of cingulate gyrus (t = −3.319, FDR-$p = 0.036$) and the bilateral thalamus proper (t = −3.168, FDR-$p = 0.036$ (left), t = −3.015, FDR-$p = 0.036$) for GM ND, and in the bilateral caudal anterior cingulate gyrus (t = −3.262, FDR-$p = 0.016$(left), t = −3.182, FDR-$p = 0.016$ (right)), the bilateral posterior cingulate gyrus (t = −3.832, FDR-$p = 0.016$ (left), t = −2.461, FDR-$p = 0.03$ (right)), the bilateral amygdala (t = −3.593, FDR-$p = 0.016$ (left), t = −3.516, FDR-$p = 0.016$ (right)) and the bilateral putamen (t = −3.228, FDR-$p = 0.016$ (left), t = −3.134, FDR-$p = 0.016$ (right)) for GM OD. The full list of statistically significant imaging features can be found in Table S1.

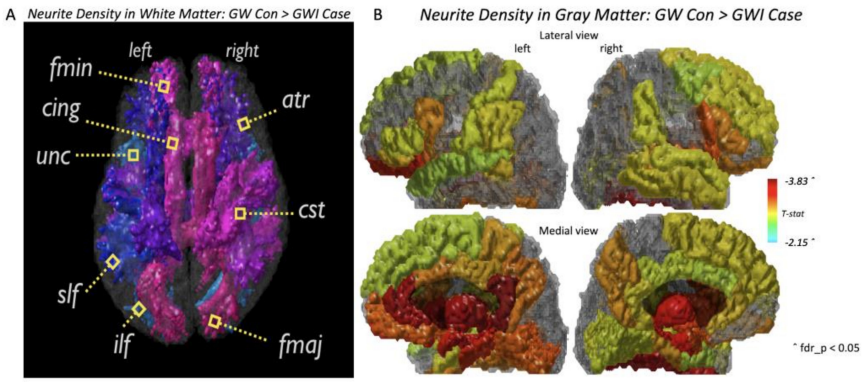

Figure 1. Gulf War illness (GWI) cases vs. Gulf War (GW) control group comparisons of gray matter (GM) and white matter (WM) neurite density imaging (NDI) measures and summary of significant regions. (**A**) 3D tract representation of significant WM ND differences between GWI case and control groups. (**B**) 3D region of interest (ROI) representation of significant GM ND differences between GWI case and control groups. Color bar corresponds to the magnitude of t-value, red indicates greater difference between groups, and vice versa. Fmaj = corpus callosum forceps major, Fmin = corpus callosum forceps minor, atr = anterior thalamic radiations, cst = corticospinal tract, cing = cingulum cingulate gyrus bundle, ilf = inferior longitudinal fasciculus, slf = superior longitudinal fasciculus, unc = uncinate fasciculus.

3.2. Machine Learning Classification Performance

As shown in Figure 2 and Table 2, the best classifier for GWI cases vs. control we had is trained using the WM OD measures, which achieved F-score of 0.941, an accuracy of 90% (SD: 0.063, highest accuracy: 91.7%), sensitivity of 95%, and specificity of 65%. The specific features include the left CST, the corpus callosum forceps minor (fminor), the left inferior fronto-occipital fasciculus (IFOF), the left inferior longitudinal fasciculus (ILF), the left superior longitudinal fasciculus (SLF), and the left superior longitudinal fasciculus temporal (SLFT). All features were statistically significant based on group-level analysis (Figure 1A, Table S1). The second-best classifier is trained using the GM ND measures, which achieved F-score of 0.922, an accuracy of 86.7% (SD: 0.054, highest accuracy:

91.7%), sensitivity of 96%, and specificity of 40%. The specific features used by this GM ND classifier include both cortical and subcortical structures of the limbic system, including the bilateral caudal anterior cingulate gyri (Table 2). The third best classifier was trained using the WM ND measures, which achieved F-score of 0.914, an accuracy of 85% (SD: 0.048, highest accuracy: 91.7%), sensitivity of 96%, and specificity of 30%. For this classifier, the specific features included the bilateral anterior thalamic radiations (ATR), the bilateral IFOF, the bilateral ILF, the left SLF, the right SLFT and the Fminor (Table 2). The full list of imaging features used by the top three classifiers can be found in Table 2 and the full list of classifier performances can be found in Table S2.

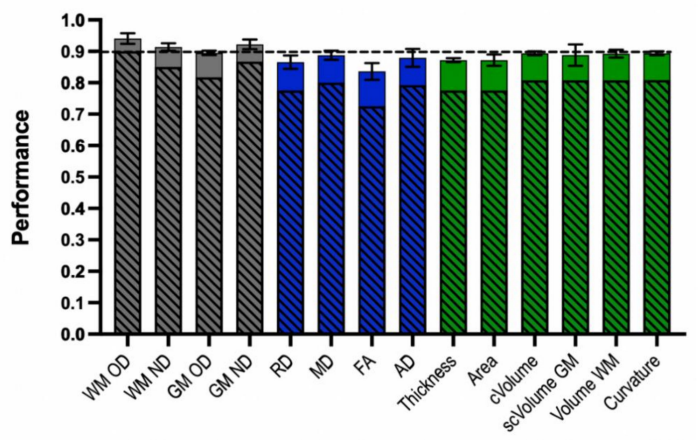

Figure 2. Classification performances of all classifiers. Each bar represents the performance (solid-colored bar: average F-score, shaded area: average accuracy) of each type of classifier trained on one imaging measure, data is presented as mean ± SEM after cross-validation. Grey-colored bars: NDI measure-based classifiers. Blue-colored bars: diffusion tension imaging (DTI) measure-based classifiers. Green-colored bars: T1-weighted structural MRI (T1W-MRI) measure-based classifiers. WM OD = white matter orientation dispersion, WM ND = white matter neurite density, GM OD = grey matter orientation dispersion, GM ND = grey matter neurite density, RD = radial diffusivity, MD = mean diffusivity, FA = fractional anisotropy, AD = axial diffusivity, thickness = cortical thickness, area = cortical surface area, cVolume = cortical volume, scVolume = subcortical GM volume, volume WM = white matter volume.

Table 2. Summary of classification performances and feature characteristics.

Measure	ACC	SEN	SPE	F-Score	Key Features	
WM OD	90%	95%	65%	0.941	L CST ** L IFOF ** L ILF ** L SLF ** L SLFT ** Fminor **	
GM ND	86.7%	96%	40%	0.922	L caudal anterior cingulate * L cuneus L inferior temporal L paracentral * L posterior cingulate * L thalamus proper *	R caudal anterior cingulat R lingual R pars orbitalis R amygdala * R putamen *
WM ND	85%	96%	30%	0.914	L ATR * L IFOF * L ILF * L SLF * Fminor *	R ATR * R IFOF * R ILF * R SLFT *

ACC: accuracy, SEN: sensitivity, SPE: specificity, F-score: F1 score, WM OD: white matter orientation dispersion index, GM ND: gray matter neurite density index, WM ND: white matter neurite density index, L: left hemisphere, R: right hemisphere, CST: corticospinal tract, IFOF: inferior fronto-occipital fasciculus, ILF: inferior longitudinal fasciculus, SLF: superior longitudinal fasciculus, SLFT: superior longitudinal fasciculus temporal, Fminor: corpus callosum forceps minor, ATR: anterior thalamic radiation. *: FDR-$p < 0.05$ in group-level statistical comparison. **: FDR-$p < 0.01$ in group-level statistical comparison.

4. Discussion

In this study, we used various neuroimaging techniques (NDI, DTI, structural T1W-MRI) to identify important features that may help to differentiate between veterans with GWI and control veterans. These features were selected through two different analytical frameworks: (1) group-level statistical analysis, and (2) single subject-level ML classification models. From our group-level, univariate analysis, we identified important imaging features, especially from WM and GM NDI and T1W-MRI regional volumetric measures, which showed high contrasts between veterans with GWI and control veterans. From the multivariate classification results, we could additionally identify unique imaging features that are important for making single-subject level inferences regardless of its relevance to the group differences.

The results from the group-level statistical analysis showed that NDI measures are the most sensitive marker for detecting GWI pathology than other types of neuroimaging measures. For WM NDI measures, all major tracts showed significant decreases for veterans with GWI compared to control veterans (Figure 1A). The greatest significant group differences were seen in the bilateral CST for WM ND and bilateral CCG bundle for WM OD (Table S1). The roles of these tracts in many essential physical and neuropsychological functions have been well described by previous literatures. For instance, earlier studies showed that disruption of the CST WM integrity was associated with motor impairment that occurs in the early stages of many neurological conditions such as Huntington's Disease and Multiple Sclerosis [38,39]. Similarly, disruption of CCG has been associated with impaired executive functioning, pain, memory deficits, and has been a main target for conditions including major depression, schizophrenia, post-traumatic stress disorder (PTSD), and autism spectrum disorder [40]. Changes in these tracts captured by our WM NDI results may also be important to understand specific symptoms such as muscle pain, fatigue, and depression observed in GWI.

From the ML framework, we confirmed that WM OD, GM ND, and WM ND measures were the sources of the top three classifiers (based on average accuracy) (Figure 2, Table 2). The classifier trained using the WM OD measure showed the best performance and consistently reporting six features: the left CST, IFOF, ILF, SLF, SLFT, and the Fminor (Table 2). Due to the completely imbalanced distribution of the data used in this study, performance on classifying controls were more challenging in QBSO and this calls better ideas on handling this issue. For example, synthetic oversampling method such as the synthetic minority oversampling technique (SMOTE) may help addressing this issue [41]. Additionally, in this type of imbalanced sample, assessing the F1-score might serve as a more realistic measure of the classification performance [37]. Although we used average accuracy measure for comparing classifiers, WM OD showed a high F-score (0.941), showing that our proposed ML framework is providing reasonable performance at least in this sample. Compared to the NDI classifiers, the classifiers from DTI measures or T1W-MRI measures all had lower classification performance than NDI measures (Table S2). These results suggest that (1) NDI measures are important imaging markers for defining GWI, and (2) the features defined from ML framework provides distinct information from the group-level statistics on describing GWI. While several features from the group-level statistics may present with overlapping patterns to ML classifiers, there are also unique features reported by ML classifiers but not captured in the group-level analysis framework.

Both our findings on group-level statistics and single subject-level classification model demonstrated the importance of NDI measures for defining GWI. Moreover, considering the other ML methods tested on mild or preclinical stage illness, such as mild cognitive impairment staying with ~78% accuracy levels, the classification performance obtained from NDI QBSO is impressive and brings more attention into the complex diffusion imaging measures for studying preclinical stage or mildly progressive illness [42]. In the current study, we not only identified widespread statistically significant NDI features through group-level analysis, but also demonstrated that WM OD measures trained a better classifier compared to other imaging measures. This is consistent with our previous studies on NDI showing that this technique is sensitive to microstructural and macrostructural brain alterations and useful for detecting neurological abnormalities in GW veterans [16]. Our result also

corroborated with our previous findings that showed a higher sensitivity for the novel NDI measures compared to the common DTI measures (e.g., FA, MD, etc.). As we suggested before, this might be due to the higher specificity of NDI for detecting changes in different tissue components [16]. We previously found that there is a strong correlation between alterations in GM ND measure and worse self-reported fatigue and sleep symptoms, and with upregulated levels of proinflammatory cytokines TNFRI and TNFRII [16]. However, based on our current findings, GM ND measures provided slightly lower classification performance than WM OD and ND measures in this study. In addition, while classifier trained on WM OD resulted in nearly identical final solutions across five iterations of validation, GM measures resulted in more variabilities in the selected feature solutions. This might be due to the differences in dimensional size between WM and GM feature space. GM measures have more numbers of features (more complexity in the feature space) to be searched out during the QBSO process than WM measures, and thereby requiring more delicate optimization process especially in this not-a-large dataset problem. Although further investigations based on larger dataset is key to address the issue, this may also indicate that WM OD measures can be better markers for simply classifying veterans with GWI from control veterans, while GM ND can be a sensitive marker to specific symptom domains. Our results also support the diagnostic value of these NDI markers for clinical applications.

Altogether, these results suggest that the microstructural changes measured by NDI may be attributed to GM and WM deficits following chronic neuroinflammation. In line with this finding, other studies have shown that chronic neuroinflammation related to GWI symptoms may be a result of both morphological and functional changes that occurred in glial cells. For instance, a study using a rat model of GWI showed that exposure to the chemical agent, diisopropyl fluorophosphate (DFP: a sarin surrogate), was associated with fewer numbers of both mature and dividing oligodendrocytes in the prefrontal cortex, which in turn interrupted the neuron-glial interactions [43]. DFP injection also induced neuroinflammation and neurodegeneration in multiple brain regions, which is associated with impaired contextual fear learning in these rats [44]. Similarly, mice exposed to DFP demonstrated epigenetic changes to genes related to the immune and neuronal systems and altered proportions of myelinating oligodendrocytes in the frontal cortex, which led to disrupted synaptic connectivity and WM alterations in GWI [45]. A recent in-vivo positron emission tomography study corroborated these findings and reported elevated levels of translocator protein (TSPO), a protein upregulated in activated microglia and astrocytes, in veterans with GWI compared to control veterans [46]. This elevation pattern was observed in many areas including the precuneus, prefrontal, primary motor, and somatosensory cortices [46]. Considering this evidence, our current findings further support the importance of novel NDI measures for detecting microstructural changes in the brain following chronic neuroinflammation in GWI.

Besides NDI measures, some T1W-MRI measures also demonstrated good performances for classifying veterans with GWI vs. control veterans. Among classifiers trained using T1W-MRI measures, the cortical volume, subcortical volume, WM volume, and mean curvature models achieved 80.8% accuracy, and highlighted key features in the frontal and temporal regions (Table S2). The results on the group-level statistical analysis also showed reduced volumes of frontal regions among veterans with GWI (Table S1). GM atrophy has been well studied as a hallmark for various neuropsychological disorders. Previous studies showed that reduced total cortical and regional frontal lobe volumes are associated with poor subjective sleep quality and increased self-reported frequency of hearing chemical alarm among GW veterans [12,47].

For DTI measures, the best performance was demonstrated by the MD classifier with an accuracy of 80% and F-score of 0.887 (Table S2). There is evidence that DTI measures may correlate with GWI symptom severity. An early study on GWI veterans showed that fatigue, pain, and hyperalgesia are associated with increased AD in the right IFOF [15]. Another study showed that changes in frontal-limbic WM connectivity, as indicated by reduced MD and increased FA in the right cingulate bundle, was associated with higher PTSD symptom severity score among a sample of 20 GW veterans [48]. In addition, GW veterans who had been exposed to chemical agents have increased

AD throughout many regions of the brain including the temporal stem, cingulum bundle, IFOF, etc., compared to unexposed veterans [13]. Through our results, we found that while T1W-MRI and DTI measures are less significant based on group-level statistical analysis, a subset of the regional measures may still explain key components of GWI symptoms.

In this study, we showed that neuroimaging markers help to identify GWI Nevertheless, we are expecting that the current approach can be improved in several aspects. One of the limitations of the current work is the imbalanced sample size, where the number of case subjects greatly exceeded the control subjects for building the classification model. This issue is reflected by the higher sensitivity and lower specificity for all the classifiers. To better handle this issue, we are planning to employ an oversampling method on the minority group to balance the samples. In our follow up work, we will also expand our analysis to a larger GW cohort including more control veterans recruited from other sites. Another important future direction is to test if the combination of multiple imaging measures, or combination of imaging and clinical measures (e.g., cognitive scores, inflammatory profiles, etc.) can improve the classification performance. This multivariate approach will be useful for identifying important features from large datasets. In conclusion, our current work provided the first evidence that novel NDI measures are not only useful for defining GWI based on the conventional group-level statistical comparisons, but also constitute key features for building single-subject level ML models for automated diagnostic classification. The features that are highlighted by our analysis suggest neurological changes underlying GWI pathology and support neuroinflammation as a potential target for therapeutic interventions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3425/10/11/884/s1, Table S1: List of key imaging features based on group-level statistical comparison, Table S2: The classification performance for all classifiers.

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